

Stimulation of Hepatocyte Glucose Metabolism by Novel Small Molecule Glucokinase Activators

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Glucokinase (GK) has a major role in the control of blood glucose homeostasis and is a strong potential target for the pharmacological treatment of type 2 diabetes. We report here the mechanism of action of two novel and potent direct activators of GK: 6-[(3-isobutoxy-5-isopropoxybenzoyl)amino]nicotinic acid (GKA1) and 5-[(3-isopropoxy-5-[2-(3-thienyl)ethoxy]benzoyl)amino]-1,3,4-thiadiazole-2-carboxylic acid (GKA2), which increase the affinity of GK for glucose by 4- and 11-fold, respectively. GKA1 increased the affinity of GK for the competitive inhibitor mannoheptulose but did not affect the affinity for the inhibitors palmitoyl-CoA and the endogenous 68-kDa regulator (GK regulatory protein [GKRP]), which bind to allosteric sites or to N-acetylglucosamine, which binds to the catalytic site. In hepatocytes, GKA1 and GKA2 stimulated glucose phosphorylation, glycolysis, and glycogen synthesis to a similar extent as sorbitol, a precursor of fructose 1-phosphate, which indirectly activates GK through promoting its dissociation from GKRP. Consistent with their effects on isolated GK, these compounds also increased the affinity of hepatocyte metabolism for glucose. GKA1 and GKA2 caused translocation of GK from the nucleus to the cytoplasm. This effect was additive with the effect of sorbitol and is best explained by a "glucose-like" effect of the GK activators in translocating GK to the cytoplasm. In conclusion, GK activators are potential antihyperglycemic agents for the treatment of type 2 diabetes through the stimulation of hepatic glucose metabolism by a mechanism independent of GKRP. *Diabetes* 53:535–541, 2004

Type 2 diabetes is a complex disease caused by defects in both the action and secretion of insulin, leading to fasting hyperglycemia and vascular complications. Current therapies do not achieve adequate control of glycemia in type 2 diabetes, and there is a growing need for novel drugs with improved efficacy to treat this disease (1).

The liver maintains glucose homeostasis in the nondiabetic state by net uptake of postprandial glucose when blood glucose exceeds the euglycemic threshold and by net production of glucose in the postabsorptive state and during fasting to maintain euglycemia. Hyperglycemia in type 2 diabetes is in part due to an altered hepatic glycemic threshold demonstrated by the lack of suppression of hepatic glucose production in the absorptive state and by excessive glucose production in the postabsorptive state (2). Enzymes that have a high control strength on hepatic glucose metabolism are potential targets for controlling hepatic glucose balance and thereby blood glucose levels in type 2 diabetes. One strong candidate in this respect is glucokinase (GK) (hexokinase IV or D), which catalyzes the first step in hepatic glucose metabolism and has a very high control strength on hepatic glucose metabolism (3). This isoform of hexokinase is expressed in pancreatic β-cells, where it is a critical component of the glucose sensory mechanism for regulation of insulin secretion (4), and in neuroendocrine cells, where it may act as a glucose sensor (5). GK differs in its kinetic properties from the other hexokinase isoforms in having a low affinity for glucose and sigmoidal kinetics, which accounts for its role as a glucose sensor (4,6).

The importance of GK in control of blood glucose homeostasis in humans is evident from the impact of mutations in the GK gene. Heterozygous loss-of-function mutations in the GK gene cause maturity-onset diabetes of the young, type 2 (7). Conversely, activating mutations cause hyperinsulinemia and hypoglycemia (8,9). Studies in rodents also support a critical role for GK in glucose homeostasis. Mice that are homozygous for disruption of the GK gene die from diabetes within days of birth, whereas the heterozygotes are hyperglycemic (10,11). Liver-specific GK overexpression in nondiabetic mice improved glucose tolerance (12,13), and in high-fat diet-induced diabetic mice, adenoviral overexpression of GK improved glucose tolerance and decreased fasting blood

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FITC, fluorescein isothiocyanate; GK, glucokinase; GKA1, 6-[(3-isobutoxy-5-isopropoxybenzoyl)amino]nicotinic acid; GKA2, 5-[(3-isopropoxy-5-[2-(3-thienyl)ethoxy]benzoyl)amino]-1,3,4-thiadiazole-2-carboxylic acid; GKRP, GK regulatory protein; MEM, minimum essential medium.

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glucose with concomitant decreased insulin secretion (14).

In the liver, GK activity is regulated by hormonal control of gene expression (15) and by a 68-kDa GK regulatory protein (GKRP), which inhibits GK competitively with respect to glucose (16). The binding affinity of GKRP for GK is increased by fructose 6-phosphate and decreased by fructose 1-phosphate, which binds to the same site on GKRP (17). GKRP also determines the subcellular location of GK and sequesters the enzyme in the nucleus in the fasted state (18). This mechanism enables rapid translocation of the enzyme to the cytoplasm in response to a rise in the extracellular concentration of glucose or fructose that occurs after a meal, resulting in a rapid increase in the rate of glucose phosphorylation (19). The role of this mechanism in regulating glucose metabolism is evident from the correlation between the rate of glucose phosphorylation and the free GK activity that is not bound to GKRP (19–21) and from the stimulation of glucose disposal by fructose administration *in vivo* (22–24). Accordingly, it can be hypothesized that agents that cause either direct activation of GK or its dissociation from GKRP (25) are a potential strategy for stimulating hepatic glucose disposal in hyperglycemic conditions.

We and others (25) have attempted to identify low-molecular weight activators of GK that may offer a novel therapeutic strategy for type 2 diabetes. A screening approach was used and compounds were selected based on activation of human GK and modulation of glucose metabolism in primary rat hepatocytes. In this study, we report the mechanism of action of two compounds, with distinct chemical structures, on GK activity and glucose metabolism in hepatocytes.

RESEARCH DESIGN AND METHODS

Expression and purification of recombinant GK and GKRP. All recombinant proteins described were cloned from rat or human cDNA using PCR. Human liver, human pancreatic, and rat liver GKs were expressed in *E. coli* with an NH₂-terminal 6His tag and purified using Ni-NTA chromatography. The protein was dialyzed in buffer A (25 mmol/L HEPES, pH 7.3, 150 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L DTT, and 5 mmol/L glucose) containing 50% glycerol and stored at -80°C. Human GKRP was expressed in *E. coli* with a COOH-terminal FLAG tag, purified by DEAE sepharose followed by M2 anti-FLAG agarose, and dialyzed in buffer A containing 20% glycerol and stored at -80°C. Recombinant proteins were >95% pure, as assessed by SDS/PAGE analysis.

Enzyme activity. GK activity was measured spectrophotometrically (340 nm, room temperature, Multiskan Ascent) in an assay mixture containing 25 mmol/L KCl, 25 mmol/L Hepes pH 7.1, 5 mmol/L β-mercaptoethanol, 1 mmol/L NADP, 1 mmol/L ATP, 2.5 mmol/L MgCl₂, 2 units/ml glucose 6-phosphate dehydrogenase, 60 nmol/L GK, and the glucose concentrations indicated. In assays containing GKRP, 200 μmol/L fructose 6-phosphate was also included. GK activity with fructose as substrate was determined as reported in Agius et al. (3) but with added phosphoglucoisomerase (10 μg/ml). For assays containing both fructose and glucose, the phosphorylation of the latter was determined radiochemically using [¹⁴C]glucose, as previously described (26). The phosphorylation of fructose was determined by subtraction of the rate of glucose phosphorylation determined radiochemically from the rate of hexose phosphorylation determined spectrophotometrically.

Hepatocyte isolation and culture. Hepatocytes were isolated by collagenase perfusion of the liver (21) of fed male Wistar rats (180–240 g body wt). They were suspended in minimum essential medium (MEM) supplemented with 7% (vol/vol) calf serum and plated in multiwell plates for metabolic studies or on gelatin-coated coverslips for immunostaining. After cell attachment (~4 h), they were cultured in serum-free MEM containing 10 nmol/L dexamethasone. Metabolic studies were performed after 16–20 h of culture.

Metabolic studies. Hepatocyte monolayers were incubated for 3 h in MEM with the concentrations of glucose and additions indicated. GK activators

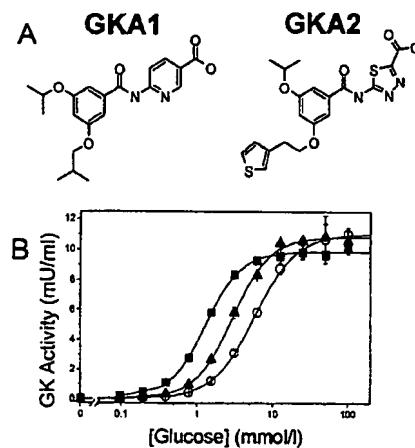


FIG. 1. GK activators increase the affinity of human GK for glucose. **A:** Chemical structures of GKA1 and GKA2. **B:** Activity of human liver GK at varying glucose concentration in the absence (○) or presence of 1 μmol/L GKA1 (▲) or 1 μmol/L GKA2 (■). A representative experiment of three summarized in Table 1.

were dissolved in DMSO, which was present at a final concentration of 0.2% (vol/vol). For determination of glucose phosphorylation or glycogen synthesis, the medium contained either [²H]glucose (0.5 mCi/ml) or [¹⁴C]glucose (1.5 mCi/ml), respectively (21). Glucose phosphorylation was determined from the formation of ³H₂O and glycolysis from the formation of lactate determined enzymatically. Glycogen synthesis from [¹⁴C]glucose was determined by ethanol precipitation (3). Free and bound GKs were determined by the digitonin release assay and free activity expressed as percentage of total activity (3). Rates of glucose metabolism were expressed as nanomoles of glucose metabolized in 3 h per milligram protein.

Size exclusion chromatography. Equimolar quantities (0.2 nmol in 50 μl) of 6His-pancreatic GK and GKRP-FLAG were incubated at room temperature for 30 min in buffer B (25 mmol/L Hepes, pH 7.3, 1 mmol/L EDTA, 1 mmol/L MgCl₂, 50 mmol/L KCl, 10 μmol/L fructose 6-phosphate, and 4 μmol/L recombinant proteins). The mixture was loaded onto a 2.4 ml Superdex200 column on a Pharmacia SMART system. The column was eluted (50 μl/min; 3 ml/60 min) with buffer B containing the indicated concentrations of fructose 1-phosphate and/or GK activator. Free GK, free GKRP, and the heterodimeric GK-GKRP complex were monitored from the absorbance peaks at 280 nm.

Immunostaining and imaging. Hepatocyte monolayers were washed in PBS and fixed in 4% paraformaldehyde in PBS (27). They were stained for GK with a rabbit IgG against human GK: 318-405 (H88, sc-7908; Santa Cruz) and FITC-labeled anti-rabbit IgG (27). Imaging for fluorescein isothiocyanate (FITC) fluorescence was performed using a Nikon Eclipse E400 epifluorescence microscope with a narrow-band filter (B2-EC). Three representative fields were selected for each condition comprising between 40 and 60 nuclei. For image capture, the maximum nuclear intensity of the control incubations was set at ~240 mean pixel intensity by adjustment of the exposure time and gain. All treatments within an experiment were captured using the settings selected for the controls. The mean pixel intensity of the nuclear and cytoplasmic areas was analyzed from the gray images using Lucia G/F analysis software. For each incubation condition, the mean value for the nuclei and the cytoplasm was determined and the results expressed as a nuclear/cytoplasmic ratio. The expression of results as a ratio corrects for drifts in intraexperimental fluorescence intensity.

RESULTS

Identification of potent activators of recombinant GK. Human GK and GKRP were used in a spectrophotometric assay adapted for a high-throughput screen to identify compounds that activate GK either directly or by causing its dissociation from GKRP. Chemical optimization of hits identified in this screening campaign generated compounds 6-((3-isobutoxy-5-isopropoxybenzoyl)amino)nicotinic acid (GKA1) and 5-((3-isopropoxy-5-[2-(3-thienyl)ethoxy]benzoyl)amino)-1,3,4-thiadiazole-2-carboxylic acid (GKA2) (Fig. 1A) for further study (28,29). GKA1 and GKA2

TABLE 1
Effects of GK activators on the affinity of GK for glucose

	$S_{0.5}$ glucose (mmol/l)	Hill coefficient	V_{max} (mU/ml)
Control	5.7 ± 0.08	1.6 ± 0.03	12.3 ± 0.45
GKA1	2.7 ± 0.11*	1.8 ± 0.02†	12.2 ± 0.75
	10 μmol/l	1.4 ± 0.08*	2.0 ± 0.03†
GKA2	1 μmol/l	1.4 ± 0.04*	1.8 ± 0.04†
	10 μmol/l	0.5 ± 0.02*	1.6 ± 0.03

Data are means ± SE for three experiments. *P < 0.001, †P < 0.01 vs. control.

increased the affinity of GK for glucose (Fig. 1B) by 2- and 4-fold, respectively, at 1 μmol/l and by 4- and 11-fold, respectively, at 10 μmol/l. There was negligible effect on V_{max} and a small increase in the Hill coefficient at 1 μmol/l (Table 1). When the effect of GKA1 on the affinity for ATP was determined at the corresponding $S_{0.5}$ for glucose, the K_m for ATP was decreased (control = 0.26 ± 0.007 mmol/l, 1 μmol/l GKA1 = 0.14 ± 0.003 mmol/l, 10 μmol/l GKA1 = 0.13 ± 0.006 mmol/l), indicating that GKA1 increases the affinity for both glucose and ATP. The potency of the compounds for activation of GK was similar when rat liver, human liver, and human β-cell GKs were compared and GKA2 was three- to fourfold more potent than GKA1 (EC₅₀ at 6 mmol/l glucose, GKA1: 1.5 ± 0.3, 1.0 ± 0.1, and 1.2 ± 0.2; GKA2: 0.4 ± 0.1, 0.3 ± 0.03, and 0.3 ± 0.01 μmol/l [means ± SE] for rat liver, human liver, and human β-cell GKs, respectively).

GK can phosphorylate various hexoses, including fructose (6). The affinity of GK for fructose is increased by low concentrations (1–4 mmol/l) of glucose (30,31). This is attributed to the effect of glucose in favoring the closed conformation (31). In this study, we confirmed that glucose (1 mmol/l) increases the affinity of GK (decrease in $S_{0.5}$) for fructose. In addition, GKA1 and GKA2 (10 μmol/l) mimicked the effect of low glucose concentration (fructose $S_{0.5}$: control, 558 ± 88 mmol/l; plus 1 mmol/l glucose, 306 ± 48 mmol/l; plus GKA1, 161 ± 15 mmol/l; plus GKA2, 143 ± 18 mmol/l [n = 3]). The Hill coefficient was unchanged by glucose (1 mmol/l) and the activators (control, 1.7 ± 0.2; glucose, 1.6 ± 0.1; GKA1, 1.7 ± 0.4; GKA2, 1.9 ± 0.3).

Effects of GKA1 on the affinity of GK for competitive inhibitors. N-acetylglucosamine, mannoheptulose, palmitoyl-CoA, and GKRP inhibit GK activity competitively with respect to glucose (6). N-acetylglucosamine and mannoheptulose are glucose analogs that are thought to bind to the catalytic site of the open and closed conformations of GK, respectively (31,32), whereas palmitoyl-CoA and GKRP bind to distinct allosteric sites. To investigate the mechanism of action of GKA1, we tested its effects on the affinity of GK for these competitive inhibitors at the corresponding $S_{0.5}$ for glucose. Activities were expressed as a percentage of controls without inhibitor (Fig. 2). GKA1 increased the affinity of GK for mannoheptulose (Fig. 2B) but did not affect the affinity for N-acetylglucosamine (Fig. 2A), palmitoyl-CoA (Fig. 2C), or GKRP (Fig. 2D). The lack of effect on the affinity for GKRP is unlikely to be an artifact of our assay conditions involving

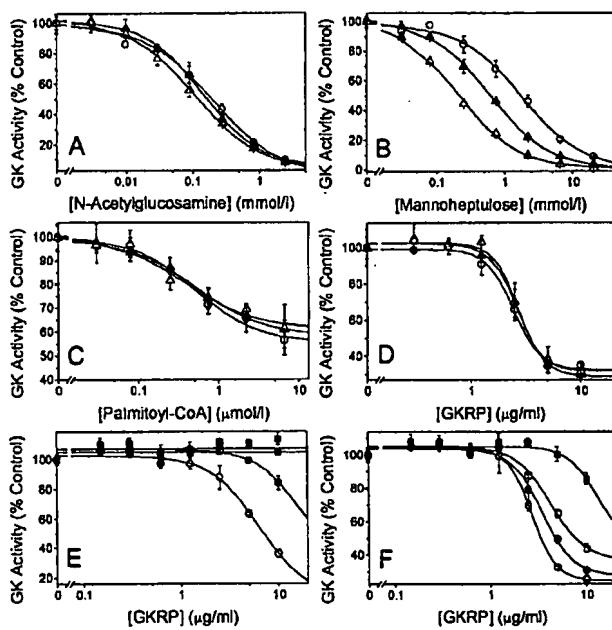


FIG. 2. GKA1 increases the affinity of GK for mannoheptulose. The affinity of GK (60 nmol/l) for N-acetylglucosamine (A), mannoheptulose (B), palmitoyl-CoA (C), and GKRP in the presence of 200 μmol/l fructose 6-phosphate (D) was determined in the absence (○) or presence of 1 μmol/l GKA1 (▲) and 10 μmol/l GKA1 (△) at the glucose concentration corresponding to the $S_{0.5}$. E and F: Effects of 10 μmol/l (●), 100 μmol/l (□), and 1 mmol/l (■) fructose 1-phosphate on the affinity for GKRP in the absence (E) or presence (F) of 200 μmol/l fructose 6-phosphate. The data are from a representative experiment (±SE) performed in triplicate.

60 nmol/l GK because fructose 1-phosphate decreased the affinity for GKRP in both the absence and presence of fructose 6-phosphate (Fig. 2E and F).

To further test the effects of GKA1 on the affinity of GKRP for GK, we used size-exclusion chromatography to separate free GK and GKRP from the heterodimeric complex. We confirmed that fructose 1-phosphate causes a concentration-dependent dissociation of the complex (Fig. 3A), as previously reported by Vandercammen and Van Schaftingen (33). However, GKA1 (10 μmol/l) did not cause dissociation of the complex (results not shown). Furthermore, when the effects of GKA1 (10 μmol/l) were tested in the presence of 10 μmol/l fructose 1-phosphate, there was also no dissociation of the complex compared with control (10 μmol/l fructose 1-phosphate), indicating that GKA1 did not potentiate dissociation of the complex in the presence of a submaximal concentration of fructose 1-phosphate (Fig. 3B). Likewise, a high glucose concentration (35 mmol/l) also did not cause dissociation of the complex (results not shown). Together, these results show that GKA1, like glucose and unlike fructose 1-phosphate, does not cause dissociation of the GK-GKRP complex and it also does not affect the inhibition of GK by GKRP.

Stimulation of glucose metabolism in hepatocytes by GKA1 and GKA2. The only known physiological activators of GK in liver are precursors of fructose 1-phosphate (fructose and sorbitol), which causes dissociation of GK from GKRP (20,34). To test the efficacy of GKA1 and GKA2 at stimulating glucose metabolism in hepatocytes, we compared their effects with sorbitol, which is taken up by

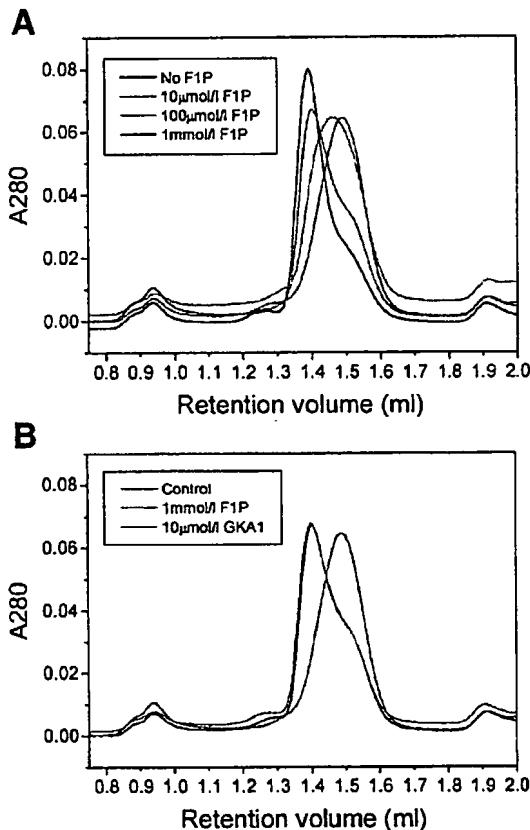


FIG. 3. GKA1 does not cause dissociation of the GKRP-GK complex. Analytical size exclusion of GK and GKRP showing separation of the individual proteins from the complex in the presence of 10 $\mu\text{mol/l}$ fructose 1-phosphate. *A*: Dissociation of the complex by increasing concentrations of fructose 1-phosphate (F1P). *B*: Dissociation of the complex by 1 $\mu\text{mol/l}$ fructose 1-phosphate but not by 10 $\mu\text{mol/l}$ GKA1 plus 10 $\mu\text{mol/l}$ fructose 1-phosphate relative to control containing 10 $\mu\text{mol/l}$ fructose 1-phosphate.

hepatocytes more rapidly than fructose and is thereby more effective at stimulating glucose metabolism (20,34). As expected, sorbitol caused a concentration-dependent increase in free GK, glucose phosphorylation, glycolysis, and glycogen synthesis (Fig. 4), with half-maximal effect at ~ 20 – $30 \mu\text{mol/l}$. GKA1 and GKA2 caused a similar increase in free GK as that achieved by maximally effective concentrations of sorbitol, and they stimulated glucose phosphorylation, glycolysis, and glycogen synthesis at all concentrations tested above 1 $\mu\text{mol/l}$, with half-maximal effect at 2–3 $\mu\text{mol/l}$ for GKA1 and 1 $\mu\text{mol/l}$ for GKA2. Although GKA1 and GKA2 caused a stimulation of glycogen synthesis that was similar to that caused by sorbitol, they caused a greater stimulation of glucose phosphorylation (Fig. 4).

GKA1 and GKA2 stimulate translocation of GK from the nucleus to the cytoplasm. To further investigate the dissociation of bound GK by GKA1 and GKA2 in hepatocytes (Fig. 4A), we tested the combined effects of the GK activators and sorbitol on dissociation of bound GK and on translocation of GK from the nucleus by immunofluorescence staining (Fig. 5). GKA1 and GKA2 had additive effects with sorbitol on dissociation of bound GK, similar to the additive effect of glucose and sorbitol (Fig. 5A).

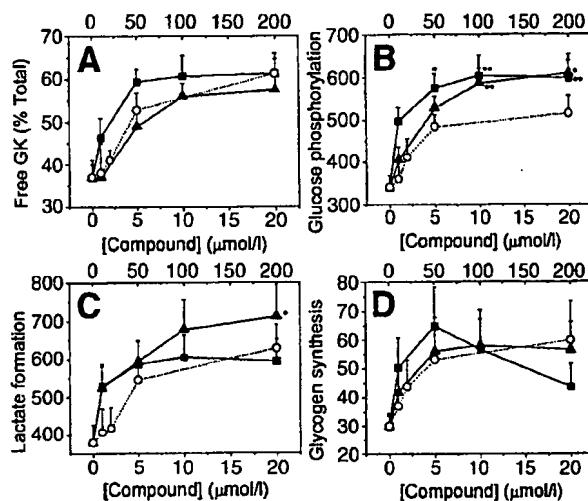


FIG. 4. Comparison of the effects of the GK activators and sorbitol on free GK activity and glucose metabolism in hepatocytes. Hepatocyte monolayers were incubated for 3 h in MEM containing 10 mmol/l glucose and the indicated concentrations of sorbitol (○; scale on top x-axis) or GKA1 (△) or GKA2 (■; scale on bottom x-axis). Free GK activity (*A*) determined by the digitonin release assay is expressed as percentage of total activity. Glucose phosphorylation (*B*), lactate formation (*C*), and glycogen synthesis (*D*) are expressed as nanmoles of glucose metabolized (*B* and *D*) or lactate formed (*C*) in 3 h/mg cell protein. The data are the means \pm SE for four (*A*), eight (*B* and *C*), and five (*D*) hepatocyte cultures. * $P < 0.05$, ** $P < 0.005$ vs. 200 $\mu\text{mol/l}$ sorbitol.

They caused translocation of GK from the nucleus, as shown by the decrease in the nuclear/cytoplasmic staining (Fig. 5B). This was additive to the effect of sorbitol, suggesting that they may act by a similar mechanism as glucose.

GKA1 increases the affinity of hepatocyte glucose metabolism for glucose. When the effect of GKA1 was tested at varying glucose concentrations, it stimulated GK dissociation, glucose phosphorylation, and glycogen synthesis at glucose concentrations up to 35 mmol/l (Fig. 6). However, the fractional stimulation was greater at low glucose (5–10 mmol/l) than at 35 mmol/l glucose (80–140 vs. 20% for glucose phosphorylation and three- to fivefold vs. 30% for glycogen synthesis). GKA1 decreased the glucose concentration that caused half-maximal stimulation from 21 ± 0.5 to 15 ± 1 mmol/l for glucose phosphorylation and from 23 ± 1 to 11 ± 1 mmol/l for glycogen synthesis.

DISCUSSION

The rate of glucose metabolism by hepatocytes is dependent on the level of expression of GK, on the molar ratio of GK/GKRP (21), and on the concentration of fructose 1-phosphate, which causes dissociation of GK from GKRP (19–21). Type 2 diabetes is associated with impaired glucose tolerance and incomplete suppression of hepatic glucose production by hyperglycemia. These defects can be corrected, in part, by fructose administration (23,24), supporting a role for GK as a potential therapeutic target for type 2 diabetes. As a first step toward a novel therapeutic strategy for the control of glycemia in type 2 diabetes, our aim was to identify potent activators of GK that increase the affinity of liver cells for glucose and

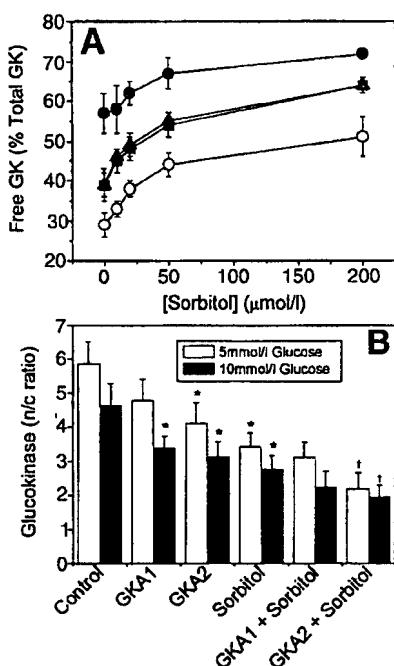


FIG. 5. GK activators have additive effects with sorbitol on GK translocation in hepatocytes. *A*: Hepatocytes were incubated for 1 h in medium containing the indicated concentrations of sorbitol in either the presence of 5 $\mu\text{mol/l}$ GKA1 (○) or 1 $\mu\text{mol/l}$ GKA2 (▲) or in the presence of 25 $\mu\text{mol/l}$ glucose (●). Free GK is expressed as a percentage of total activity. *B*: Hepatocytes were incubated for 1 h with sorbitol (200 $\mu\text{mol/l}$) and/or the GK activators (10 $\mu\text{mol/l}$) in medium with either 5 or 10 $\mu\text{mol/l}$ glucose. The nuclear/cytoplasmic (n/c) ratio of GK was determined by immunofluorescence staining. The data are the means \pm SE for four to five cultures. * $P < 0.05$ vs. corresponding glucose control; † $P < 0.05$ vs. corresponding sorbitol.

thereby lower the hepatic glucose threshold. Although there are various known competitive inhibitors of GK, both physiological and pharmacological (6,16), there are as yet no clearly established physiological activators of GK other than fructose 1-phosphate, which indirectly activates GK by causing its dissociation from GKRP. We report here the structures of two chemically distinct direct activators of GK that potently stimulate hepatic glucose metabolism with a generally similar profile as physiologically

cal activation by precursors of fructose 1-phosphate but by a mechanism that is independent of GKRP.

GKA1 and GKA2 increase the affinity of human GK for glucose by 4- to 10-fold with an EC_{50} of 0.3–1 $\mu\text{mol/l}$. The mechanism by which GKA1 increases the affinity for glucose was investigated by testing its effects on the affinity of GK for various competitive inhibitors. GKA1 did not affect the affinity for *N*-acetylglucosamine, which binds to the catalytic site (35), or for palmitoyl-CoA and GKRP, which bind to allosteric sites (6). However, it increased the affinity for mannoheptulose, which is thought to bind to the catalytic site (31,32). This glucose analog differs from *N*-acetylglucosamine in its effects on the cooperativity of GK for glucose (36). This has been explained by binding of these inhibitors to the open (*N*-acetylglucosamine) or closed (mannoheptulose) conformations of GK (31,32). Further insight into the mechanism of action of the GK activators can be envisaged from recent studies on the kinetic properties of GK mutants. Mutations of residues in the catalytic site that interact with glucose affect the affinity for glucose, *N*-acetylglucosamine, and mannoheptulose (37–39). However, mutations of residues distant from the catalytic site affect the affinity for glucose and mannoheptulose but not *N*-acetylglucosamine (31,37). Most, though not all, of the activating mutations are in the hinge region of the bipartite structure (8,9,32,38). It is assumed that they favor the closed conformation, which has a higher affinity for glucose. The effects of GKA1 in increasing the affinity for glucose and mannoheptulose, but not *N*-acetylglucosamine, are very similar to those of activating mutations. Therefore, we propose that GKA1 binds to GK at a site distinct from both the catalytic site and the allosteric sites for palmitoyl-CoA and GKRP. Occupancy of this allosteric activation site would favor the high-affinity closed conformation. It remains speculative whether this site has a physiological ligand or binds glucose analogs.

The efficacy of a pharmacological activator of GK would be dependent on its cellular efficacy. It was crucial, therefore, to confirm that the GK activators stimulate glucose metabolism in hepatocytes. We show that GKA1 and GKA2 are potent activators (EC_{50} of 1 $\mu\text{mol/l}$ for GKA2 and ~2–3 $\mu\text{mol/l}$ for GKA1) of glucose metabolism in hepatocytes. The similarity in the effects of the activators

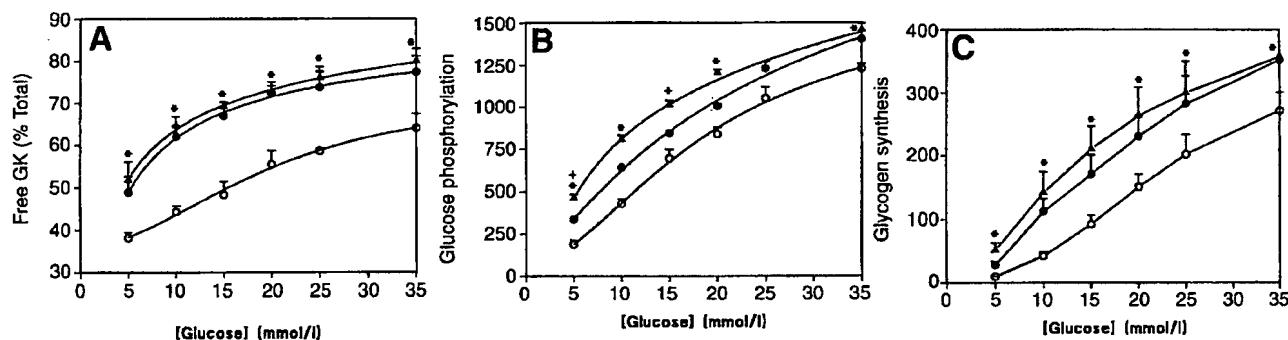


FIG. 6. Effects of GKA1 at varying glucose concentration. Hepatocytes were incubated for 3 h in medium with the glucose concentrations indicated in either the absence (○) or presence of 50 $\mu\text{mol/l}$ sorbitol (●) or 10 $\mu\text{mol/l}$ GKA1 (▲). Glucose phosphorylation (*B*) and glycogen synthesis (*C*) are expressed as nanomoles of glucose metabolized in 3 h per milligram cell protein. The data are the means \pm SE for four to five cultures. * $P < 0.05$, GKA1 vs. corresponding control; † $P < 0.05$, GKA1 vs. corresponding incubations with sorbitol.

and sorbitol suggests that the compounds are selective for GK and do not appear to affect downstream sites.

The stimulation of glucose metabolism by sorbitol is explained by the fructose 1-phosphate-induced dissociation of GK from GKRP and its translocation to the cytoplasm (18–20). The finding that the GK activators caused dissociation of bound GK and translocation to the cytoplasm appeared incongruous with the size exclusion data showing that GKA1 does not dissociate GK from GKRP. High glucose concentration and glucose analogs that are not metabolized by GK to a significant extent (5-thioglucose and mannoheptulose) also cause translocation of GK from the nucleus (18,27,36). It has been generally assumed that the translocation induced by glucose is due to dissociation of GK from GKRP. However, we confirmed by size exclusion chromatography that high glucose, like GKA1 and unlike fructose 1-phosphate, does not cause dissociation of GK from GKRP. We propose the following hypothesis to account for these observations. Translocation of GK from the nucleus to the cytoplasm may require two complementary mechanisms: release of GK from GKRP, which is localized predominantly but not exclusively in the nucleus (18,27), and transfer to another GK receptor that is localized predominantly in the cytoplasm (3). Various GK binding proteins have been identified by the yeast two-hybrid system (40–42) or by random peptide library screening (41), which are potential candidates for the cytoplasmic receptor. The synergistic effects of glucose, as well as precursors of fructose 1-phosphate (36), on GK translocation would then be explained by the latter promoting GK release from GKRP and the former promoting its binding to cytoplasmic receptors. Therefore, the action of the GK activators on translocation would be similar to the effect of glucose. Consistent with such a “glucose-like” mechanism of action, we have also shown that like glucose, GK activators increase the affinity of GK for fructose.

Despite the general similarity in the effects of the GK activators and sorbitol, the net stimulation of glucose phosphorylation by GKA1 and GKA2 was greater than that by sorbitol. This can be explained by the combined effects of the GK activators on translocation of GK and on activation of GK. Likewise, the increase in the affinity of glucose phosphorylation and glycogen synthesis in hepatocytes for glucose by GKA1 can be explained by the combined effects on translocation and activation.

A recent study by Grimsby et al. (25) reported activation of GK by RO-28-1675. In our study, we report the structures of two novel potent activators of human GK that increase the affinity for glucose. Moreover, we demonstrate the efficacy of these compounds in stimulating glucose phosphorylation, glycolysis, and glycogen synthesis in hepatocytes. Furthermore, we show that the compounds affect the affinity for mannoheptulose but not for three other GK inhibitors, including GKRP, which suggests a similar mechanism of action of the compounds to activating mutations of the GK gene (8,9,32). The compounds cause translocation of GK from the nucleus despite the fact that they do not dissociate GK from GKRP. We explain this by our finding that high glucose concentration does not cause dissociation of the GK-GKRP complex. This supports the hypothesis for a glucose-like effect of the compounds on GK translocation.

In the nondiabetic state, the liver maintains glucose homeostasis by net uptake or net production of glucose relative to the prevailing glycemia. This mechanism is impaired in type 2 diabetes when an excessive production of glucose by the liver is a major contributor to fasting hyperglycemia. GK activators that stimulate net uptake of glucose by the liver by increasing the affinity of the liver cell for glucose would therefore shift the balance from net glucose output to net uptake and would be predicted to lower blood glucose, as has been shown by activating mutations of the GK gene in humans. GK activators may help in the development of novel treatments for type 2 diabetes. In addition, they are powerful experimental tools that may shed new insights into our understanding of control of glucose homeostasis by GK.

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